NUCLEIC ACID ENZYME BIOSENSORS FOR IONS

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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BACKGROUND

Many metals pose a risk as environmental contaminants. A well-known example is lead. Low level lead exposure can lead to a number of adverse health effects, with as many as 9-25% of pre-school children presently at risk. The level of lead in the blood considered toxic is ≥ 10 μg/dL (480 nM). Current methods for lead analysis, such as atomic absorption spectrometry, inductively coupled plasma mass spectrometry, and anodic stripping voltammetry, often require sophisticated equipment, sample pre-treatment, and skilled operators.

Simple, rapid, inexpensive, selective and sensitive methods that permit real time detection of Pb^{2+} and other metal ions are very important in the fields of environmental monitoring, clinical toxicology, wastewater treatment, and industrial process monitoring. Furthermore, methods are needed for monitoring free or bioavailable, instead of total, metal ions in industrial and biological systems.

Fluorescence spectroscopy is a technique well suited for very small concentrations of analytes. Fluorescence provides significant signal amplification, since a single fluorophore can absorb and emit many photons, leading to strong signals even at very low concentrations. In addition, the fluorescence time-scale is fast enough to allow real-time monitoring of concentration fluctuations. The fluorescent properties only respond to changes related to the fluorophore, and therefore can be highly selective. Furthermore, fluorometers for uses in the field are commercially available. Fluorescent detection is also compatible with fiber-optic technology and well suited for *in vivo* imaging applications. Several fluorescence-related parameters can be assessed for the purpose of sensing, including fluorescence intensity, emission or excitation wavelength, fluorescence lifetime and anisotropy.

Many fluorescent chemosensors, including fluorophore-labeled organic chelators (Rurack, *et al.*, 2000; Hennrich *et al.*, 1999; Winkler *et al.*, 1998; Oehme & Wolfbeis, 1997) and peptides (Walkup & Imperiali, 1996; Deo & Godwin, 2000; Pearce *et al.*, 1998), have been developed for metal ion detection. These ion sensors are usually composed of an ion-binding motif and a fluorophore. Metal detection using these fluorescent chemosensors relies on the modulation of the fluorescent properties of the fluorophore by the metal-binding event. Detection limits on the level of micromolar and even nanomolar concentrations have been achieved for heavy metal ions including Zn^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+} and Ag^{+}. The design and synthesis of a chemosensor that exhibits highly selective and sensitive binding of the metal ion of choice in aqueous solution is still a big challenge, although the metal binding and the fluorescent moieties of the sensor can be systematically varied to achieve desired properties.

Nucleic acid molecules have previously been adapted to sense the presence of nucleic acids and to detect gene mutations from inherited diseases or chemical damages. In recent years, the molecular recognition and catalytic function of nucleic acids have been extensively explored. This exploration has lead to the development of aptamers and nucleic acid enzymes.

Aptamers are single-stranded oligonucleotides derived from an *in vitro* evolution protocol called systematic evolution of ligands by exponential enrichment (SELEX). Nucleic acid aptamers have been isolated from random sequence pools and can selectively bind to non-nucleic acid targets, such as small organic molecules or proteins, with affinities as high as 10^{-14} M (Uphoff *et al.*, 1996; Famulok, 1999). Most aptamers undergo a conformational change when binding their cognate ligands. With this property, several DNA and RNA aptamers have been engineered to sense L-adenosine or thrombin through an internally labeled fluorescent reporter group (Jhaveri *et al.*, 2000). Thus, the conformational change in the aptamer upon binding leads to a change in fluorescence.

Nucleic acid enzymes are nucleic acid molecules that catalyze a chemical reaction. *In vitro* selection of nucleic acid enzymes from a library of 10^{14}-10^{15} random nucleic acid sequences offers considerable opportunity for developing enzymes with desired characteristics (Breaker & Joyce, 1994; Breaker, 1997). Compared with

combinatorial searches of chemo- and peptidyl-sensors, *in vitro* selection of DNA/RNA is capable of sampling a larger pool of sequences, amplifying the desired sequences by polymerase chain reactions (PCR), and introducing mutations to improve performance by mutagenic PCR.

Allosteric ribozymes (or aptazymes), which combine the features of both aptamer and catalytic RNA, also hold promises for sensing small molecules (Potyrailo *et al.*, 1998; Koizumi *et al.*, 1999; Robertson & Ellington, 1999, 2000). Their reactivity is modulated through the conformational changes caused by the binding of small organic molecules to an allosteric aptamer domain. Therefore, the signal of ligand binding can be transformed into a signal related to chemical reaction.

Divalent metal ions can be considered as a special class of cofactors controlling the activity of nucleic acid enzymes. The reaction rate of the nucleic acid enzymes depends on the type and concentration of the metal ion in solution. Several RNA and DNA enzymes obtained through *in vitro* selection are highly specific for Cu^{2+}, Zn^{2+}, and Pb^{2+}, with metal ion requirements on the level of micromolar concentrations (Breaker & Joyce, 1994; Pan & Uhlenbeck, 1992; Carmi *et al.*, 1996; Pan et al., 1994; Cuenoud & Szotak, 1995; Li *et al.*, 2000; Santoro *et al.*, 2000).

BRIEF SUMMARY

The present invention uses nucleic acid enzymes as signal transducers for ion detection. Compared with fluorescent chemosensor and protein biosensors, nucleic acid-based sensors are more amenable to combinatorial search for sequences with desired metal specificity and affinity. In addition, DNA, in particular, is stable and can be readily synthesized. A wide range of fluorescent dyes can be easily introduced at specific sites to suit different needs. DNA-based biosensors can also be adapted for use with optical fiber and DNA-chip technology for applications such as *in vivo* imaging, *in situ* detection, and array sensing.

In one aspect, the present invention provides for specific and sensitive biosensors of ions. The biosensors are useful in methods of detecting the presence of

an ion, particularly metal ions such as Pb^{2+}. In certain embodiments, the biosensors may be used to determine the concentration of a particular ion in a solution.

The biosensors of the present invention use nucleic acid enzymes that require the presence of specific ions for their activity. Enzymatic activity leads to hydrolytic cleavage of a substrate nucleic acid that may be part of the nucleic acid enzyme itself. The resulting cleavage product then may be detected indicating the presence of the ion.

In a preferred embodiment, the biosensor comprises a fluorophore and a quencher arranged in proximity such that prior to cleavage the fluorescence intensity is decreased by the quencher. However, upon cleavage, the fluorophore and quencher are separated leading to an increase in fluorescence intensity. In a further preferred embodiment, the biosensor contains an array of nucleic acid enzymes having a range of sensitivities and specificities to several different ions.

A "nucleic acid enzyme" is a nucleic acid molecule that catalyzes a chemical reaction. The nucleic acid enzyme may be covalently linked with one or more other molecules yet remain a nucleic acid enzyme. Examples of other molecules include dyes, quenchers, proteins, and solid supports. The nucleic acid enzyme may be entirely made up of ribonucleotides, deoxyribonucleotides, or a combination of ribo- and doxyribonucleotides.

A "sample" may be any solution that may contain an ion (before or after pre-treatment). The sample may contain an unknown concentration of an ion. For example, the sample may be paint that is tested for lead content. The sample may be diluted yet still remains a sample. The sample may be obtained from the natural environment, such as a lake, pond, or ocean, an industrial environment, such as a pool or waste stream, a research lab, common household, or a biological environment, such as blood. Of course, sample is not limited to the taking of an aliquot of solution but also includes the solution itself. For example, a biosensor may be placed into a body of water to measure for contaminants. In such instance, the sample may comprise the body of water or a particular area of the body of water. Alternatively, a solution may be flowed over the biosensor without an aliquot being taken. Furthermore, the sample

may contain a solid or be produced by dissolving a solid to produce a solution. For example, the solution may contain soil from weapon sites or chemical plants.

"Measuring the product of the nucleic acid enzymatic reaction" includes measuring the result of the production of a product by an enzyme. For example, in an embodiment where the substrate comprises a quencher and the enzyme comprises a fluorophore and cleavage of the substrate by the enzyme leads to dissociation of the product from the enzyme, "measuring the product" includes detecting the increase of fluorescence. Thus, one is measuring the product by detecting its inability to quench fluorescence.

"Contacting a nucleic acid enzyme with a sample" includes placing the sample and enzyme in proximity such that an ion in the sample could be used as a cofactor. "Contacting" includes such acts as pipetting a sample onto a solid support or into a tube or well containing the nucleic acid enzyme. Alternatively, the enzyme may be brought to the sample. For example, the enzyme may be placed into a stream to monitor for the presence of a contaminant.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Selection scheme for RNA-cleaving deoxyribozymes. **FIG. 1A.** Starting pool of random-sequenced DNAs, engineered to contain two substrate-binding domains. Each member of the pool contains a 5'-terminal biotin (encircled B), a single embedded ribonucleotide (rA) and a 40-nucleotide random sequence domain (N40). **FIG. 1B.** Selective amplification scheme for isolation of DNA that catalyzes the metal cofactor (Co^{2+} or Zn^{2+}) dependent cleavage of an RNA phosphodiester.

FIG. 2. Sequence classes of the cloned Zn-DNA. The numbers on the left are the clone-numbers randomly assigned to the sequences during the cloning and sequencing process. The highly conserved sequences (Region-20nt) are in bold. The covariant nucleotides are underlined. The 5'- and the 3'-primer binding sequences are shown in italic.

FIG 3. Sequence classes of the cloned Co-DNA. The clone-numbers are listed on the left. The 5' and the 3' primer binding sequences are in italic.

FIG. 4. Sequence alignment of the N40 region of the reselected Zn-DNAs. The wild-type sequence is listed on the top, followed by the reselected Zn-DNA sequences. Only the point mutations are shown for the reselected sequences, while the nucleotides that are identical to the wild type at the corresponding positions are omitted. Numbers listed on the left are clone-numbers. The rate constants (k_{obs}) of several reselected Zn-DNA in 100 μM Zn^{2+} are shown on the right.

FIG. 5. Proposed secondary structure of the Zn(II)-dependent *trans*-cleaving deoxyribozyme.

FIG. 6. Sequences and proposed secondary-structures of several RNA-cleaving deoxyribozymes. **FIG. 6A and FIG. 6B.** The deoxyribozyme selected using Mg^{2+} or Pb^{2+} as cofactor (Breaker & Joyce, 1994, 1995). **FIG. 6C and FIG. 6D.** The 10–23 and the 8–17 deoxyribozymes selected in Mg^{2+} to cleave all-RNA substrate (Santoro & Joyce, 1997). **FIG. 6E.** A deoxyribozyme selected using L-histidine as cofactor. **FIG. 6F.** The 17E deoxyribozyme selected in Zn^{2+}. In each structure, the upper strand is the substrate and the lower strand is the enzyme. Arrows identify the site of RNA transesterification.

FIG. 7. Comparison of G3 deoxyribozyme with class II Co-DNA. **FIG. 7A.** The predicted secondary structure of the G3 deoxyribozyme (Geyer & Sen, 1997). X represents variable sequences. The boxed region was also found in class II Co-DNA. **FIG. 7B.** The minimal structure motif of the class II Co-DNA predicted by *mfold* program. The arrows indicate the cleavage sites.

FIG. 8. Steady-state fluorescence spectra of the substrate (Rh-17DS) alone (I), after annealing to the deoxyribozyme (17E-Dy) (II), and 15 min after adding 500 nM $Pb(OAc)_2$ (III).

FIG. 9. The fluorescence response rate (vfluo) of Rh-17EDS-Dy for different divalent metal ions. The "control" was measured without Pb(II) or transition metal ions. **FIG. 9A.** with 500 nM M(II) in 50 mM HEPES (pH 7.5); The inserted graph

shows the change of fluorescence intensity at 580 nm in response to the addition of M(II). The curve with dramatic change was collected in Pb(II), the other curves were collected in one of the other eight divalent metal ions. **FIG. 9B.** with 500 nM M(II) in 100 mM NaCl, 1 mM Mg^{2+}, 1 mM Ca^{2+} and 50 mM HEPES (pH 7.5).

FIG 10. Dependence of v_{fluo} on the concentration of Pb^{2+} or Co^{2+}. The reaction was carried out in the presence of 50 mM NaCl in 50 mM HEPES (pH 7.5). **FIG 10A.** The initial rate (v_{fluo}) increased with the concentration of Pb^{2+} (◆) and Co^{2+} (■) over a range of three orders of magnitude. **FIG 10B**. At low concentrations, v_{fluo} increased linearly with Pb^{2+} (◆) or Co^{2+} (■) concentration.

FIG. 11. DNA chips for ion sensing. **FIG. 11A.** The array of deoxyribozymes with different metal specificity and affinity on the DNA chip for metal ion sensing. **FIG. 11B.** Quantitative and qualitative detection of metal ions using the metal ion-sensing deoxyribozyme chip. The z-axis represents the fluorescence intensity change upon the exposure of the chip to the sample under examination. The change in the fluorescence intensity is caused by the deoxyribozyme-catalyzed substrate cleavage in the presence of a specific kind and concentration of metal ion.

DETAILED DESCRIPTION

The invention described herein represents a new class of ion sensors and is the first example of a DNA enzyme-based biosensor for ions. It combines the high selectivity of DNA enzymes with the high sensitivity of fluorescence detection. For example, in one embodiment, selectivity for Pb^{2+} was > 80 fold over other divalent metal ions with high sensitivity (> 400% signal increase). Such selectivity and sensitivity provides for qualitative and quantitative detection of ions over a concentration range of several orders of magnitude. In a preferred embodiment, the fluorescence domain is decoupled from the ion-recognition/catalysis domain, and therefore the sensitivity and selectivity of this system may be manipulated by a careful choice of fluorophores and by performing *in vitro* selection of ion-binding domains to not only keep sequences reactive with the ion of choice, but also remove sequences that also respond to other ions.

In addition, DNA is stable, inexpensive and easily adaptable to optical fiber and chip technology for device manufacture. The attachment of DNA enzymes to optical fibers or chips allows regeneration of the sensors by washing away the cleavage products and adding new substrates. Finally, sequences specific for other ions and with various detection ranges may be isolated by varying the selection conditions, providing for a highly sensitive and selective fluorosensor system.

Nucleic Acid Enzymes

A growing number of nucleic acid enzymes have been discovered or developed showing a great diversity in catalytic activity (Table 1 and Table 2). Many if not all of the enzymes are dependent on one or more ion cofactors. *In vitro* selection may be used to "enhance" selectivity and sensitivity for a particular ion. Such enzymes find particular utility in the compositions and methods of the present invention. For example, nucleic acid enzymes that catalyze molecular association (ligation, phophorylation, and amide bond formation) or dissociation (cleavage or transfer) are particularly useful.

In preferred embodiments, a nucleic acid enzyme that catalyzes the cleavage of a nucleic acid in the presence of an ion is used. The nucleic acid enzyme may be RNA (ribozyme), DNA (deoxyribozyme), a DNA/RNA hybrid enzyme, or a peptide nucleic acid (PNA) enzyme. PNAs comprise a polyamide backbone and the bases found in naturally occurring nucleosides and are commercially available from, e.g., Biosearch, Inc. (Bedford, Mass.).

Ribozymes that may be used in the present invention include, but are not limited to, group I and group II introns, the RNA component of the bacterial ribonuclease P, hammerhead, hairpin, hepatitis delta virus and Neurospora VS ribozymes. Also included are *in vitro* selected ribozymes, such as those isolated by Tang and Breaker (2000).

One limitation of using a ribozyme is that they tend to be less stable than deoxyribozymes. Thus, in preferred embodiments, the nucleic acid enzyme is a deoxyribozyme. Preferred deoxyribozymes include those shown in FIG. 6A-6F and deoxyribozymes with extended chemical functionality (Santoro *et al.*, 2000).

Table 1.

Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments.

Reaction	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/k_{uncat} [a]	Reference
Phosphoester centers				
Cleavage	0.1	0.03	10^5	Vaish, 1998
Transfer	0.3	0.02	10^{13}	Tsang, 1996
Ligation	100	9	10^9	Ekland, 1995
Phosphorylation	0.3	40	$>10^5$	Lorsch, 1994
Mononucleotide polymerization	0.3	5000	$>10^7$	Ekland, 1996
Carbon centers				
Aminoacylation	1	9000	10^6	Illangasekare, 1997
Aminoacyl ester hydrolysis	0.02	0.5	10	Piccirilli, 1992
Aminoacyl transfer	0.2	0.05	10^3	Lohse, 1996
N-alkylation	0.6	1000	10^7	Wilson, 1995
S-alkylation	4×10^{-3}	370	10^3	Wecker, 1996
Amide bond cleavage	1×10^{-5}		10^2	Dai, 1995
Amide bond formation	0.04	2	10^5	Wiegand, 1997
Peptide bond formation	0.05	200	10^6	Zhang, 1997
Diels-Alder cycloaddition	>0.1	>500	10^3	Tarasow, 1997
Others				
Biphenyl isomerization	3×10^{-5}	500	10^2	Prudent, 1994
Porphyrin metallation	0.9	10	10^3	Conn, 1996

[a] Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments. kcat/kuncat is the rate enhancement over uncatalyzed reaction.

Table 2.
Deoxyribozymes isolated through *in vitro* selection.

Reaction	Cofactor	$k_{max}(min^{-1})$[a]	k_{cat}/k_{uncat}	Reference
RNA transesterification	Pb^{2+}	1	10^5	Breaker, 1994
	Mg^{2+}	0.01	10^5	Breaker, 1995
	Ca^{2+}	0.08	10^5	Faulhammer, 1997
	Mg^{2+}	10	$>10^5$	Santoro, 1997
	None	0.01	10^8	Geyer, 1997
	L-histidine	0.2	10^6	Roth, 1998
	Zn^{2+}	~40	$>10^5$	Li, J., 2000
DNA cleavage	Cu^{2+}	0.2	$>10^6$	Carmi, 1996
DNA ligation	Cu^{2+} or Zn^{2+}	0.07	10^5	Cuenod, 1995
DNA phosphorylation	Ca^{2+}	0.01	10^9	Li, Y., 1999
5',5'-pyrophophate formation	Cu^{2+}	5×10^{-3}	$>10^{10}$	Li, Y., 2000
Porphyrin metalation	None	1.3	10^3	Li, Y., 1996

[a] k_{max} is the maximal rate constant obtained under optimized conditions.

An advantage of ribozymes and deoxyribozymes is that they may be produced and reproduced using biological enzymes and appropriate templates. However, the present invention is not limited to ribozymes and deoxyribozymes. Nucleic acid enzymes that are produced by chemical oligosynthesis methods are also included. Thus, nucleic acids including nucleotides containing modified bases, phosphate, or sugars may be used in the compositions and methods of the present invention. Modified bases are well known in the art and include inosine, nebularine, 2-aminopurine riboside, N^7-denzaadenosine, and O^6-methylguanosine (Earnshaw & Gait, 1998). Modified sugars and phosphates are also well known and include 2'-deoxynucleoside, abasic, propyl, phosphorothioate, and 2'—O-allyl nucleoside (Earnshaw & Gait, 1998). DNA/RNA hybrids and PNAs may be used in the

compositions and methods of the present invention. The stability of PNAs and relative resistance to cellular nucleases make PNA enzymes amenable to *in vivo* applications.

In certain embodiments, the substrate for the nucleic acid enzyme and the enzyme itself are contained in the same nucleic acid strand. Such enzymes are *cis*-acting enzymes. Examples include the Zn^{2+}-dependent deoxyribozymes (Zn-DNA) created in Example 1 (FIG. 1A and FIG. 2).

In preferred embodiments, the nucleic acid enzyme cleaves a nucleic acid strand that is separate from the strand comprising the enzyme (*trans*-acting). One advantage of utilizing *trans*-activity is that, after cleavage, the product is removed and additional substrate may be cleaved by the enzymatic strand. A preferred nucleic acid enzyme is 5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3' (17E; FIG. 5; SEQ ID NO:1). The corresponding preferred substrate to 17E is *5'*-ACTCACTATrAGGAAGAGATG-3' (17DS; FIG. 5; SEQ ID NO:2), where rA denotes a single ribonucleotide.

It may be beneficial to use directed mutation to change one or more properties of a nucleic acid enzyme or its substrate. Using 17E and 17DS as an example, one may wish to alter the avidity of the two arms of the hybridized enzyme and substrate. The "arms" are those areas displaying Watson-Crick basepairing in FIG. 5. To alter avidity, one may increase or decrease the length of the arms. Increasing the length of the arms increases the number of Watson-Crick bonds, thus increasing the avidity. The opposite is true for decreasing the length of the arms. Decreasing the avidity of the arms facilitates the removal of substrate from the enzyme, thus allowing faster enzymatic turnover.

Another method of decreasing avidity includes creating mismatches between the enzyme and the substrate. Alternatively, the G-C content of the arms may be altered. Of course, the effect of any directed change should be monitored to ensure that the enzyme retains its desired activity, including ion sensitivity and selectivity. In light of the present disclosure, one of skill in the art would understand how to monitor for a desired enzymatic activity. For example, to ensure that the mutated

enzyme maintained sensitivity and selectivity for Pb^{2+}, one would test to determine if the mutated enzyme remained reactive in the presence of lead (sensitivity) and maintained its lower level of activity in the presence of other ions (selectivity).

In preferred embodiments, the nucleic acid enzyme is sensitive and selective for a single ion. The ion may be any anion or cation. The ion may be monovalent, divalent, trivalent, or polyvalent. Examples of monovalent cations include K^+, Na^+, Li^+, Tl^+, NH_4^+, and Ag^+. Examples of divalent cations include Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}, Pt^{2+}, Ra^{2+}, Ba^{2+}, and Sr^{2+}. Examples of trivalent cations include Co^{3+}, Cr^{3+}, and lanthanide ions (Ln^{3+}). Polyvalent cations include Ce^{4+}, spermine, and spermidine. Because, in certain embodiments, the biosensors of the present invention are used to monitor contaminants in the environment, preferred ions are those that are toxic to living organisms, *e.g.*, Ag^+, Pb^{2+} and Hg^{2+}.

Often the nucleic acid enzymes that have activity with one ion also have at least some activity with one or more other ions. Such multi-sensitive enzymes may still be used in the compositions and methods of the present invention. However, it should be understood that use of a multi-sensitive enzyme may lead to uncertainty as to which of the ions is present. In such cases, measuring the rate of enzymatic activity, using serial dilutions, or using an array of nucleic acid enzymes may be helpful in deciphering which ion is present.

In vitro Selection of Nucleic Acid Enzymes

Many nucleic acid enzymes that are dependent on ions, particularly metal ions, for activity are known in the art (Breaker & Joyce, 1994; Pan & Uhlenbeck, 1992; Cuenoud & Szostak, 1995; Carmi *et al.*, 1996; Li *et al.*, 2000; Santoro *et al.*, 2000). In light of the present disclosure, one of skill in the art would understand how to utilize a known nucleic acid enzyme in the methods and biosensors of the present invention. Furthermore, the present invention may include a nucleic acid enzyme created by *in vitro* selection. Methods of *in vitro* selection of nucleic acid enzymes are known in the art and described herein.

In vitro selection is a technique in which RNA or DNA molecules with certain functions are isolated from a large number of sequence variants through multiple cycles of selection and amplification (Joyce, 1994; Chapman *et al.*, 1994). The concept of *in vitro* selection of catalytic RNA molecules was first introduced in the late 1980's. Since then, it has been widely applied to obtain ribozymes with maximized activities or novel catalytic abilities, and to identify oligonucleotides (called aptamers) that bind to certain proteins or small molecules with high affinity. The process for aptamers selection is sometimes referred as systematic evolution of ligands by exponential enrichment (SELEX)(Tuerk & Gold, 1990).

The first catalytic DNA (deoxyribozyme) was isolated by Breaker and Joyce in 1994 through *in vitro* selection. This deoxyribozyme is able to catalyze phosphodiester cleavage reaction in the presence of Pb^{2+}. Unlike RNA-based catalysts, DNA molecules with catalytic functions have not been encountered in nature, where DNA exists primarily as base-paired duplex and serves mainly as the carrier of genetic information. The identification of DNA molecules with catalytic functions further demonstrated the power of *in vitro* selection.

In vitro selection is typically initiated with a large collection of randomized sequences. A typical DNA or RNA library for selection contains 10^{13}-10^{16} sequence variants. The construction of a completely randomized pool is accomplished by chemical synthesis of a set of degenerated oligonucleotides using standard phosphoramidite chemistry. The 3'-phosphoramidite compounds of four nucleosides (A, C, G, and T) are premixed before being supplied to an automated DNA synthesizer to produce oligonucleotides. By controlling the ratio of four phosphoroamidites, the identity at each nucleotide position can be either completely random, i.e. with equal chance for each base, or biased toward a single base. Other strategies for creating a randomized DNA library include applying mutagenic polymerase chain reaction (PCR) and template-directed mutagenesis (Tsang and Joyce, 1996; Cadwell and Joyce, 1992, 1994). For the purpose of *in vitro* selection of functional RNA molecules, the randomized DNA library is converted to an RNA library through *in vitro* transcription.

In vitro selection takes advantage of a unique property of RNA and DNA, i.e., the same molecule can possess both genotype (coding information) and phenotype (encoded function). The DNA or RNA molecules in the randomized library are screened simultaneously. Those sequences that exhibit a desired function (phenotype) are separated from the inactive molecules. Usually the separation is performed through affinity column chromatography, being linked to or released from a solid support, gel electrophoresis separation, or selective amplification of a tagged reaction intermediate. The genotype of the active molecules are then copied and amplified, normally through polymerase chain reaction (PCR) for DNA or isothermal amplification reaction for RNA (Guatelli *et al.*, 1990). Mutations can be performed with mutagenic PCR to reintroduce diversity to the evolving system. These three steps — selection, amplification and mutation, are repeated, often with increasing selection stringency, until sequences with the desired activity dominate the pool.

Novel nucleic acid enzymes isolated from random sequences *in vitro* have extended the catalytic repertoire of RNA and DNA far beyond what has been found in nature. The selected ribozymes are capable of catalyzing a wide range of reactions at both phosphate and non-phosphate centers (Table 1). The reactions that are catalyzed by deoxyribozymes are less diverse, compared with the ribozymes (Table 2). However, the catalytic rate (k_{cat}) of most deoxyribozymes is comparable to that of the ribozymes catalyzing the same reaction. In certain cases, the catalytic efficiency (k_{cat}/K_m) of nucleic acid enzymes even exceeds that of the protein enzymes.

In vitro selection can be used to change the ion specificity or binding affinity of existing ribozymes, or to obtain nucleic acid enzymes specific for desired ions. For example, *in vitro*-selected variants of the group I intron (Lehman & Joyce, 1993) and the RNase P ribozyme (Frank & Pace, 1997) have greatly improved activity in Ca^{2+}, which is not an active metal ion cofactor for native ribozymes. The Mg^{2+} concentration required for optimal hammerhead ribozyme activity has been lowered using *in vitro* selection to improve the enzyme performance under physiological conditions (Conaty *et al.*, 1999; Zillman et al., 1997). Breaker and Joyce have isolated several RNA-cleaving deoxyribozymes using Mg^{2+}, Mn^{2+}, Zn^{2+}, or Pb^{2+} as

the cofactor (Breaker & Joyce, 1994, 1995). Only the sequence and structure of the Pb^{2+}-dependent and the Mg^{2+}-dependent deoxyribozymes were reported (FIG. 6A and 6B). Other examples of metal-specific RNA/DNA enzymes obtained through *in vitro* selection include a Pb^{2+}-specific RNA-cleaving ribozyme (called leadzyme)(Pan & Uhlenbeck, 1992), a Cu^{2+}-specific DNA-cleaving deoxyribozyme (Carmi *et al.*, 1996), and a DNA ligase active in Zn^{2+} and Cu^{2+} (Cuonod & Szostak, 1995).

Often nucleic acid enzymes developed for a specific metal ion by *in vitro* selection will have activity in the presence of other metal ions. For example, 17E deoxyribozyme was developed by *in vitro* selection for activity in the presence of Zn^{2+}. Surprisingly, the enzyme showed greater activity in the presence of Pb^{2+} than Zn^{2+}. Thus, although produced in a process looking for Zn^{2+}-related activity, 17E may be used as a sensitive and selective sensor of Pb^{2+}.

To produce nucleic acid enzymes with greater selectivity, a negative selection step may be included in the process. For Example, Pb^{2+}-specific deoxyribozymes may be isolated using a similar selection scheme as for the selection of Co^{2+}- and Zn^{2+}-dependent DNA enzymes described in Example 1. In order to obtain deoxyribozymes with high specificity for Pb^{2+}, negative-selections may be carried out in addition to the positive selections in the presence of Pb^{2+}.

For negative selection, the DNA pool is selected against a "metal soup", which contains various divalent metal ions (e.g. Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, etc.). Those sequences that undergo self-cleavage in the presence of divalent metal ions other than Pb^{2+} are then washed off the column. The remaining sequences are further selected with Pb^{2+} as the cofactor. Pb^{2+}-dependent deoxyribozymes with different affinities for Pb^{2+} can be obtained by controlling the reaction stringency (Pb^{2+} concentration).

Fluorophores and Quenchers

Any chemical reaction that leads to a fluorescent or chemiluminescent signal may be used in the compositions and methods of the present invention. In preferred embodiments, fluorophores are used to measure enzymatic activity and, thus, detect

the presence of a particular ion. Essentially any fluorophore may be used, including BODIPY, fluoroscein, fluoroscein substitutes (Alexa Fluor dye, Oregon green dye), long wavelength dyes, and UV-excited fluorophores. These and additional fluorphores are listed in Fluorescent and Luminescent Probes for Biological Activity. A Practical Guide to Technology for Quantitative Real-Time Analysis, Second Ed. W.T. Mason, ed. Academic Press (1999) (incorporated herein by reference). In preferred embodiments, the fluorophore is 6-carboxytetramethylrhodamin (TAMRA). TAMRA has an excitation range of 500-550 nm and an emission range of 560-650 nm.

In certain embodiments, the substrate is labeled with a fluorophore and measurement of enzymatic activity is done by detecting the non-hybridized cleavage products in solution. Preferably, this is done by measuring the level of fluorescence in solution without detecting fluorescence from the bound substrate. This may be done by creating a flow such that, once the cleavage product enters the solution, it is carried away by the flow. Fluorescence of the flow is then measured in an area away from the enzyme-substrate pairs.

In preferred embodiments, the substrate is labeled with a fluorophore but fluorescence is quenched by a nearby quenching molecule. Quenching molecules absorb the energy of the excited fluorophore. Close proximity of fluorophore and quencher allow for the energy to be transferred from the fluorophore to the quencher. By absorbing this energy, the quencher prevents the fluorophore from releasing the energy in the form of a photon.

Quenchers may be categorized as non-fluorescent and fluorescent quenchers. Non-fluorescent quenchers are capable of quenching the fluorescence of a wide variety of fluorophores. Generally, non-fluorescent quenchers absorb energy from the fluorophore and release the energy as heat. Examples of non-fluorescent quenchers include DABCYL, QSY-7, and QSY-33.

Fluorescent quenchers tend to be specific to fluorophores that emit at a specific wavelength range. Fluorescent quenchers often involve fluorescence resonance energy transfer (FRET). In many instances the second molecule is also a

fluorophore. In such cases, close proximity of the fluorophore and quencher is indicated by a decrease in fluorescence of the "fluorophore" and an increase in fluorescence in the fluorescent quencher. Commonly used fluorescent fluorophore pairs (fluorophore/fluorescent quencher) include fluorescein/tetramethylrhodamine, IAEDANS/fluorescein, fluorescein/fluorescein, and BODIPY FL/ BODIPY FL.

The quencher may be located on a support such that it is in proximity with the fluorophore when the substrate is bound to the enzyme. In preferred embodiments, the quencher is linked to the enzyme. Even more preferred is to have the fluorphore linked to the 5' end of the substrate and the quencher linked to the 3' end of the enzyme such that when the substrate and enzyme are hybridized, the fluorophore and the quencher are in close proximity to each other. Upon cleavage of the substrate, the product disassociates from the enzyme. Dissociation removes the fluorophore from the quencher, leading to an increase in fluorescence (FIG. 8).

Of course, it would be understood that the fluorophore could be linked essentially anywhere on the substrate and quencher essentially anywhere on the enzyme, as long as they are in close proximity to each other when the enzyme is hybridized to the substrate. By close proximity, it is meant that they are situated such that the quencher is able to function. Furthermore, the quencher may be placed on the substrate and the fluorophore on the enzyme. Alternatively, both quencher and fluorophore may be linked to the substrate on opposite ends from the potential cleavage site. Cleavage of such a molecule would lead to dissociation of the two ends and thus separation of the fluorophore and quencher, leading to an increase in fluorescence. Similarly, in embodiments wherein the enzyme and the substrate are contained within the same nucleic acid strand, the fluorophore and quencher may be placed on opposite ends of the cleavage site.

When choosing a fluorophore, quencher, or where to position the molecules, it is important to consider, and preferably to test, the effect of the fluorphore or quencher on the enzymatic activity of the nucleic acid enzyme. Also, it is preferable that the fluorophore display a high quantum yield and energy transfer efficiency. Long-wavelength (excitation and emission) fluorophores are preferred because of less

interference from other absorbing species. The fluorophore should also be less sensitive to pH change or to non-specific quenching by metal ions or other species.

Methods and devices for detecting fluorescence are well developed. Essentially any instrument or method for detecting fluorescent emissions may be used. For example, WO 99/27351 (incorporated herein in its entirety) describes a monolithic bioelectronical device comprising a bioreporter and an optical application specific integrated circuit (OASIC). The device allows remote sampling for the presence of substances in solution.

Furthermore, the fluorescence may be measured by a number of different modes. Examples include fluorescence intensity, lifetime, and anisotropy in either steady state or kinetic rate change modes (Lakowicz, 1999).

Sometimes other factors in a solution such as pH, salt concentration or ionic strength, or viscosity will have an effect on fluorescence. Others may affect the hybridization of the substrate and enzyme. Therefore, in preferred methods, controls are run to determine if the solution itself, regardless of enzymatic activity, is altering the fluorescence. Such controls include the use of non-cleavable substrates and or substrate without the presence of enzyme.

Biosensors

Described herein are nucleic acid enzymes that are dependent on the presence of a specific ion for activity. Using fluorophores or fluorophore/quencher labeling, it is possible to measure enzymatic activity, even in real time. These qualities make the compositions of the present invention excellent for use in biosensors for detecting ions.

Many biosensors utilizing nucleic acids are known in the art. For example, biosensors using aptamers have been developed for detecting molecules such as thrombin or adenosine (Potyrailo *et al.*, 1999; Lee & Walt, 2000). In light of the present disclosure, one of ordinary skill in the art would know how to modify the nucleic acid biosensors to include nucleic acid enzymes.

In a simple embodiment, a biosensor of the present invention comprises a nucleic acid enzyme labeled with a quencher, a substrate labeled with a fluorophore, and a device to detect fluorescence such as a fluorescence microscope or a fluorometer. In a method using this embodiment, the enzyme and substrate are contacted with a sample suspected of containing an ion to which the enzyme is sensitive. Fluorescence is measured and compared to a control wherein the ion is absent. Change in fluorescence is indicative of the presence of the ion.

Of course, many variants of even this simple embodiment are included within the scope of the invention. Such variants include placing the enzyme, substrate, and sample in the well of a microtiter plate and measuring fluorescence with a microtiter plate reader. In another variation, the enzyme is attached to a solid support. When the enzyme is attached to a solid support, it is preferable that a linker is used. An exemplary linking system is biotin/streptavidin. For example, the biotin molecule may be linked to the enzyme and a plate may be coated with streptavidin. When linking an enzyme to a solid support, it is important to determine the effect of linkage on the enzymatic activity of the enzyme.

In an alternative embodiment, the solid support may be a bead and fluorescence measured using a flow cytometer. In embodiments having the enzyme attached to a solid support, the biosensor may be reusable. Old substrate and sample is removed, leaving the enzyme in place. New substrate and sample may then be added.

In another embodiment, the nucleic acid enzyme may be used in conjunction with fiber-optics (Lee & Walt, 2000). The nucleic acid enzyme may be immobilized on the surface of silica microspheres and distributed in microwells on the distal tip of an imaging fiber. The imaging fiber may then be coupled to a epifluorescence microscope system.

In certain embodiments, the biosensor will comprise an array of nucleic acid enzymes. The arrays of the present invention provide for the simultaneous screening of a variety of ion by nucleic acid enzymes. The array may contain as little as 2 or as many as 10,000 different nucleic acid enzymes. Of course, any integer in between

may be used. Preferably, each individual nucleic acid enzyme has a measurable difference in specificity or affinity for at least one ion compared to at least one other nucleic acid enzyme within the array.

In preferred embodiments, the array is a high-density array like those used in DNA-chip technologies. Methods of forming high density arrays of nucleic acids with a minimal number of synthetic steps are known (U.S. Pat. No. 6,040,138). The nucleic acid array can be synthesized on a solid support by a variety of methods, including light-directed chemical coupling, and mechanically directed coupling (U.S. Pat. No. 5,143,854; WO 90/15070; WO 92/10092; WO 93/09668). Using this approach, one heterogenous array of polymers is converted, through simultaneous coupling at a number of reaction sites, into a different heterogenous array.

The light-directed combinatorial synthesis of nucleic acid arrays on a glass surface uses automated phosphoramidite chemistry and chip masking techniques. In one specific implementation, a glass surface is derivatizied with a silane reagent containing a functional group, e.g., a hydroxyl or amine group blocked by a photolabile protecting group. Photolysis through a photolithogaphic mask is used selectively to expose functional groups which are then ready to react with incoming 5'-photoprotected nucleoside phosphoramidites. The phosphoramidites react only with those sites which are illuminated (and thus exposed by removal of the photolabile blocking group). Thus, the phosphoramidites only add to those areas selectively exposed from the preceding step. These steps are repeated until the desired array of sequences have been synthesized on the solid surface. Combinatorial synthesis of different nucleic acid analogues at different locations on the array is determined by the pattern of illumination during synthesis and the order of addition of coupling reagents.

In the event that a PNA is used in the procedure, it is generally inappropriate to use phosphoramidite chemistry to perform the synthetic steps, since the monomers do not attach to one another via a phosphate linkage. Instead, peptide synthetic methods are substituted (U.S. Pat. No. 5,143,854).

In addition to the foregoing, additional methods which can be used to generate an array of nucleic acids on a single solid support are known (For example, WO 93/09668). In these methods, reagents are delivered to the solid support by either (1) flowing within a channel defined on predefined regions or (2) "spotting" on predefined regions. However, other approaches, as well as combinations of spotting and flowing, may be employed. In each instance, certain activated regions of the solid support are mechanically separated from other regions when the monomer solutions are delivered to the various reaction sites.

A typical "flow channel" method applied to the nucleic acid enzyme arrays of the present invention can generally be described as follows. Diverse nucleic acid sequences are synthesized at selected regions of a solid support by forming flow channels on a surface of the solid support through which appropriate reagents flow or in which appropriate reagents are placed. For example, assume a monomer "A" is to be bound to the solid support in a first group of selected regions. If necessary, all or part of the surface of the solid support in all or a part of the selected regions is activated for binding by, for example, flowing appropriate reagents through all or some of the channels, or by washing the entire solid support with appropriate reagents. After placement of a channel block on the surface of the solid support, a reagent having the monomer A flows through or is placed in all or some of the channel(s). The channels provide fluid contact to the first selected regions, thereby binding the monomer A on the solid support directly or indirectly (via a spacer) in the first selected regions.

Thereafter, a monomer B is coupled to second selected regions, some of which may be included among the first selected regions. The second selected regions will be in fluid contact with a second flow channel(s) through translation, rotation, or replacement of the channel block on the surface of the solid support; through opening or closing a selected valve; or through deposition of a layer of chemical or photoresist. If necessary, a step is performed for activating at least the second regions. Thereafter, the monomer B is flowed through or placed in the second flow channel(s), binding monomer B at the second selected locations. In this particular

example, the resulting sequences bound to the solid support at this stage of processing will be, for example, A, B, and AB. The process is repeated to form a vast array of nucleic acid enzymes of desired length and sequence at known locations on the solid support.

After the solid support is activated, monomer A can be flowed through some of the channels, monomer B can be flowed through other channels, a monomer C can be flowed through still other channels, etc. In this manner, many or all of the reaction regions are reacted with a monomer before the channel block must be moved or the solid support must be washed and/or reactivated. By making use of many or all of the available reaction regions simultaneously, the number of washing and activation steps can be minimized.

One of skill in the art will recognize that there are alternative methods of forming channels or otherwise protecting a portion of the surface of the solid support. For example, according to some embodiments, a protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) is utilized over portions of the solid support to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

The "spotting" methods of preparing nucleic acid arrays can be implemented in much the same manner as the flow channel methods. For example, a monomer A can be delivered to and coupled with a first group of reaction regions which have been appropriately activated. Thereafter, a monomer B can be delivered to and reacted with a second group of activated reaction regions. Unlike the flow channel embodiments described above, reactants are delivered by directly depositing (rather than flowing) relatively small quantities of them in selected regions. In some steps, of course, the entire solid support surface can be sprayed or otherwise coated with a solution. In preferred embodiments, a dispenser moves from region to region, depositing only as much monomer as necessary at each stop. Typical dispensers include a micropipette to deliver the monomer solution to the solid support and a

robotic system to control the position of the micropipette with respect to the solid support. In other embodiments, the dispenser includes a series of tubes, a manifold, an array of pipettes, or the like so that various reagents can be delivered to the reaction regions simultaneously.

Methods of detecting fluorescent signals on a DNA chip are well known to those of skill in the art. In a preferred embodiment, the nucleic acid enzyme array is excited with a light source at the excitation wavelength of the particular fluorescent label and the resulting fluorescence at the emission wavelength is detected. In a particularly preferred embodiment, the excitation light source is a laser appropriate for the excitation of the fluorescent label.

A confocal microscope may be automated with a computer-controlled stage to automatically scan the entire high density array. Similarly, the microscope may be equipped with a phototransducer (e.g., a photomultiplier, a solid state array, a ced camera, etc.) attached to an automated data acquisition system to automatically record the fluorescence signal produced by each nucleic acid enzyme on the array. Such automated systems are described at length in U.S. Pat. No: 5,143,854 and PCT application 20 92/10092.

EXAMPLES

The following examples are included to demonstrate embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain like or similar results without departing from the spirit and scope of the invention.

Example 1 – *In vitro* selection of a ion-dependent deoxyribozyme

This example demonstrates a method of creating nucleic acid enzymes that are dependent on the presence of an ion for activity. More specifically, use of a partially

random DNA library to obtain deoxyribozymes that cleave RNA in the presence of Zn^{2+} or Co^{2+} is shown.

Materials and Methods used in this Example

Oligonucleotides

DNA oligonucleotides were purchased from Integrated DNA Technologies Inc. Sequences of the random DNA template and the primers (P1, P2 and P3) used in PCR amplifications are listed below:

P1: 5'-GTGCCAAGCTTACCG-3' (SEQ ID NO:3)

P2: 5'-CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3' (SEQ ID NO:4)

P3: 5'-GGGACGAATTCTAATACGACTCACTATrA-3' (SEQ ID NO:5)

Template for random DNA pool:

5'-GTGCCAAGCTTACCGTCAC-N40-GAGATCTCGCCATCTCTTCCT ATAGTGAGTCGTATTAG-3' (SEQ ID NO:6)

Primer P1b and P3b are the 5'-biotinylated version of primers P1 and P3. Primer P1a and P3a were prepared by 5'-labeling P1 and P3 with [γ-^{32}P] ATP (Amersham) and T4 polynucleotide kinase (Gibco). The DNA/RNA chimeric substrate (17DS) for *trans*-cleavage assays has the sequence 5'-ACTCACTATrAGGAAGAGATG-3' (SEQ ID NO:2), where rA denotes a single ribonucleotide. The all-RNA substrate (17RS) with the same sequence was purchased from Dharmacon Research Inc. The *trans*-cleaving deoxyribozyme 17E has the sequence 5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3' (SEQ ID NO:1). The deoxyribozyme named 17E1 is a variant of 17E with the sequence 5'-CATCTCTTTTGTCAGCGACTCGAAATAGTGA GT-3' (SEQ ID NO:7). All oligonucleotides were purified using denaturing polyacrylamide gel electrophoresis and desalted with the SepPak nucleic acid purification cartridges (Waters) before use.

Preparation of Random DNA Pool

The initial pool for DNA selection was prepared by template-directed extension followed by PCR amplification. The extension was carried out with 200 pmol of

DNA template containing a 40-nucleotide random sequence region, and 400 pmol of primer P3b in 20× 100 µl reaction mixtures for four thermal-cycles (1 min at 92°C, 1 min at 52°C, and 1 min at 72°C). Reaction buffer also included 0.05 U/µl *Taq* polymerase (Gibco), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 0.01% gelatin and 0.2 mM of each dNTP. Subsequently, 1 nmol each of P1 and P3b were added to the extension product to allow four more cycles of PCR amplification. The products were precipitated with ethanol and dissolved in 0.5 ml of buffer A, which contains 50 mM HEPES (pH 7.0), 500 mM (for Zn-DNA selection) or 1 M (for Co-DNA selection) NaCl. About 20 µM EDTA was also added to the buffer to chelate trace amount of divalent metal ion contaminants.

In Vitro Selection

The random DNA pool was immobilized on a NeutrAvidin column (Pierce) by incubating with the column materials for 30 minutes. The mixture was gently vortex-mixed a few times during the incubation. The unbound DNA strands were eluted with at least 5× 100 µl of buffer A. The non-biotinylated strands of immobilized DNA were washed off the column with 5× 100 µl of freshly prepared 0.2 M NaOH and 20 µM EDTA. The column was then neutralized with 5× 100 µl of buffer A. The cleavage reaction was carried out by incubating the immobilized single-stranded DNA containing the single ribonucleotide (rA) with 3× 20 µl of reaction buffer (buffer A plus 1 mM $ZnCl_2$ or $CoCl_2$) over 1h. The eluted DNA molecules were pooled and precipitated with ethanol. A fraction of the selected DNA was amplified in 100 µl PCR reaction with 40 pmol each of primers P1 and P2 over 10–20 thermal cycles. One tenth of the PCR product was further amplified for six cycles with 50 pmol of primers P1 and P3b. The final PCR product was ethanol precipitated and used to initiate the next round of selection. During the selection of Zn(II)-dependent deoxyribozymes (called Zn-DNA hereafter), the concentration of $ZnCl_2$ was kept constant at 100 µM in the reaction buffer for the following rounds of selection. Reaction time was gradually decreased from 1 h to 30 s within 12 rounds of selection. For the selection of Co(II)-dependent deoxyribozymes (called Co-DNA hereafter), the

concentration of $CoCl_2$ was gradually decreased from 1 mM to 100 μM and the reaction time from 1 h to 1 min within 10 rounds of selection. The twelfth generation of selectively amplified Zn-DNA and the tenth generation of Co-DNA were cloned using TA–TOPO Cloning Kit (Invitrogen) and sequenced with T7 Sequenase 2.0 Quick-denatured Plasmid Sequencing Kit (Amersham).

Reselection

Based on the sequence of class I Zn-DNA or Co-DNA, partially degenerate DNA template libraries for reselection were synthesized (Integrated DNA Technology Inc.) with 20% degeneracy at the N40 region. In other words, during the oligonucleotide synthesis of the N40 region, the wild type sequence was introduced at a probability of 80% at each position, while the other three nucleotides each occurred at a probability of 6.67%. The reselection pool was prepared with 10 pmol of template and 100 pmol of primers P1 and P3b using the same protocol previously described. With 10 pmol (number of molecules $S = 6 \times 10^{12}$) of partially randomized template, the statistic parameters of the DNA library used for reselection were calculated based on the following equations.

$$P(k,n,d) = [n!/(n-k)!k!]d^k(1-d)^{n-k} \quad (1)$$

$$N(k) = [n!/(n-k)!k!]3^k \quad (2)$$

$$C(n,k) = SP(k,n,d)/N(k) \quad (3)$$

P(k,n,d) is the probability of having k mutations within n (number of randomized positions, n = 40) nucleotide positions that have been randomized at a degeneracy of d. N(k) is the number of distinct sequences that have k mutations with respect to the prototype sequence. C(n,k) is the number of copies for each sequence that has k mutations. The reselection pool was expected to contain the wild type sequence, all possible sequences with 1–8 point mutations, and a sampling of the sequences with >8 point mutations. More than half of the population contains ≥8 point-mutations. The protocol for reselection was the same as the primary selection, except that the reaction time was decreased from 20 min to 1 min and the concentration of $ZnCl_2$ or $CoCl_2$ was decreased from 20 μM to 5 μM over six

generations. The sixth generation of reselected Zn- or Co-DNA were cloned and sequenced as previously described.

Kinetic Assays of the Reselected *Cis*-cleaving DNA

The 5' ^{32}P-labeled precursor DNA for *cis*-cleavage assay was prepared by PCR-amplification of the selected DNA population or the cloned DNA plasmid with primer 1b and 3a. The double-stranded product was immobilized on a NeutrAvidin column through the biotin moiety on primer P1b. The catalytic strand of DNA was eluted off the column with 3× 20 µl freshly prepared 0.2 N NaOH and neutralized with 8 µl of 3 M sodium acetate (pH 5.3) in the presence of 50 µg/ml bovine serum albumin (Sigma). Following ethanol precipitation, the single-stranded DNA was purified on an 8% denaturing polyacrylamide gel and desalted with SepPak nucleic acid purification cartridge. Bovine serum albumin (50 µg/ml) was added to the gel-soaking buffer (0.2 M NaCl, 20 µM EDTA, 10 mM Tris–HCl, pH 7.5) to prevent the DNA from adhering to the tube. The concentration of the DNA was determined by scintillation counting the radioactivity.

The precursor DNA was dissolved in buffer A and incubated at room temperature for 10 min before $CoCl_2$ or $ZnCl_2$ was added. The reaction was stopped with 50 mM EDTA, 90% formamide and 0.02% bromophenol blue. Reaction products were separated on an 8% denaturing polyacrylamide gel and quantified with a Molecular Dynamic phosphorimager.

In Vitro Selection of Zn(II)- or Co(II)-dependent Deoxyribozymes

The DNA molecules capable of cleaving an RNA bond in the presence of Co^{2+} or Zn^{2+} were obtained through *in vitro* selection. The initial DNA library for selection contains ~10^{14} out of the possible 10^{24} (= 4^{40}) DNA sequences. These molecules consist of a random sequence domain of 40 nucleotides flanked by two conserved primer-binding regions. The sequence of the conserved region was designed in such a way that they could form two potential substrate-binding regions (FIG. 1A). A ribonucleic adenosine was embedded in the 5'-conserved sequence region and was intended to be the cleavage site, since an RNA bond is more susceptible than a DNA

bond toward hydrolytic cleavage. The intrinsic half-life of the phosphodiester linkage in RNA at pH 7 and 25°C is estimated to be 1,000 years. The corresponding value for DNA is 200 million years.

The DNA pool was immobilized on a NeutrAvidin column through the biotin moiety on the 5' terminus of the DNA. Biotin and Avidin bind strongly with an association constant of $K_a = 10^{15}$ M^{-1}. The sequences that underwent self-cleavage in the presence of Co^{2+} or Zn^{2+} were eluted off the column, amplified and used to seed the next round of selection (FIG. 1B). The selection stringency was increased during the selection process with shorter reaction time and less available divalent metal ions. The activity of the selected Zn-DNA gradually increased until the twelfth generation and declined thereafter, while the highest activity was achieved with the tenth generation of Co-DNA. Therefore the twelfth generation of Zn-DNA and the tenth generation of Co-DNA were cloned and sequenced. The cloned sequences can be divided into different classes based on sequence similarity (FIG. 2 and FIG. 3).

Individual sequences of the cloned Zn-DNA and Co-DNA were randomly chosen and sampled for activity. Under the selection conditions (100 μM Zn^{2+}, 500 mM NaCl, 50 mM HEPES, pH 7.0, 25°C), the observed rate constants of Zn-DNAs from sequence-classes I and II were 0.1–0.2 min^{-1}, while class III sequences were less active, with k_{obs} around 0.02 min^{-1}. The cleavage rate of the initial pool was 2×10^{-7} min^{-1}. Therefore, a 10^5–10^6 fold increase in cleavage rate has been achieve for Zn-DNA selection. The cleavage rates of all the randomly picked Co-DNA sequences were <0.02 min^{-1} under the conditions for Co-DNA selections (100 μM Co^{2+}, 1 M NaCl, 50 mM HEPES, pH 7.0, 25°C). Interestingly, even in the buffer (1 M NaCl, 50 mM HEPES, pH 7.0) alone, the class II Co-DNA exhibited similar activity as in the presence of 100 μM Co^{2+} or Zn^{2+}.

Clone #5 of Zn-DNA (Zn-5) and clone #18 of Co-DNA (Co-18) showed relatively high activity, as well as high frequency of occurrence, within their lineages. The k_{obs} were 0.17 min^{-1} for Zn-5 (in 100 μM Zn^{2+}) and 0.02 min^{-1} for Co-18 (in 100 μM Co^{2+}). The sequences of Zn-5 and Co-18 were partially randomized (see Material

and Methods for details) and subjected to reselection in order to further improve the reactivity and metal-binding affinity, and to explore the sequence requirement of the conserved catalytic motif. Based on equations (1) – (3), the reselection pool was expected to contain the wild type sequence, all possible sequences with 1–8 point mutations, and a sampling of the sequences with >8 point mutations. More than half of the population should contain ≥8 point mutations. Six rounds of reselection were carried out with 5–20 μM Zn^{2+}or Co^{2+}, however the activity of the reselected DNA was similar to the activity of the wild type sequences. Sequencing of the Zn-DNA from both the initial selection and reselection revealed a highly conserved sequence region. Therefore the lack of activity improvement after reselection likely reflects a sequence pool dominated by a few highly reactive sequences.

Sequence Alignment and Structure Analysis of Zn-DNA

The sequences of thirty individual clones of initially selected Zn-DNA can be divided into three major classes based on sequence similarity. Differences among members of each class were limited to a few point mutations (FIG. 2). A highly conserved sequence region of 20 nt, 5'-$TX_1X_2X_3AGCY_1Y_2Y_3TCGAAATAGT$-3' (SEQ ID NO:8) (Region-20nt), was observed in all but one sequence albeit at different locations. The sequences of 5'-$X_1X_2X_3$-3' and 3'-$Y_3Y_2Y_1$-5' are complimentary and covariant, indicating that they form base pair with each other:

5'-$X_1X_2X_3$-3'

3'-$Y_3Y_2Y_1$-5'

The secondary structures of the sequenced Zn-DNA were predicted using Zuker's *DNA mfold* program (see http://mfold.wustl.edu/~folder/dna/form1.cgi) through minimization of folding energy. The most stable structures predicted for those containing Region-20nt all contained a similar structure motif. This common motif consists of a pistol-shaped three-way helical junction formed by a 3 bp hairpin, an 8 bp hairpin and a double helix linking to the rest of the molecule. The 3 bp hairpin and its adjacent single-stranded regions are part of the Region-20nt. The ribonucleic adenosine is unpaired and positioned opposite of the 3 bp hairpin.

After reselection, twenty-eight Zn-DNA clones were sequenced (FIG. 4). When compared with the parental wild type sequence (class I Zn-DNA), the reselected Zn-DNA contained point mutations mostly outside of Region-20nt. About one third of these sequences have a T → A mutation at position 73, converting the T–T mismatch in the wild type sequence to a Watson–Crick base pair. In one fourth of the reselected DNAs, the 5 nucleotide single-stranded bulge of the three-way junction has the sequence 5'-ACGAA-3', corresponding to 5'-TCGAA-3' in the wild type. Clone #17 (named ZnR17) of the reselected Zn-DNA is most active under selection conditions (FIG. 4). Structural analysis of ZnR17 revealed two completed base-paired helices in the three-way junction. Therefore, it was engineered into a *trans*-cleaving deoxyribozyme by deleting the sequences outside of the three-way junction and the loop of the 8 bp hairpin. Such truncation resulted in two individual stands, which hybridize with each other through two 9-10 bp helices. The strand containing the single ribonucleotide residue (rA) is considered as the substrate (named 17DS), while the other strand as the enzyme (named 17E). The catalytic core, which was highly conserved during selection, consists of a 3 bp hairpin and a 5 nt single-stranded bulge (FIG. 5).

Although ZnR17 was selected in Zn^{2+}, it does not contain structure motifs that were discovered in several Zn(II)-binding RNA molecules (Ciesiolka *et al.*, 1995; Ciesiolka & Yarus, 1996). However, the conserved region of ZnR17 is very similar to that of the 8–17 deoxyribozymes selected by Santoro and Joyce using Mg^{2+} as cofactor (Santoro & Joyce, 1997). The unpaired bulge region in the 8–17 DNA enzyme has the sequence 5'-WCGR-3' or 5'-WCGAA-3' (W = A or T; R = A or G). The highest activity was observed with the sequence containing 5'-TCGAA-3'. Among the Zn(II)-dependent deoxyribozymes we obtained after reselection, 85% of them fell within the 5'-WCGAA-3' regime (W = A or T). However, the sequence of the two double helices flanking the catalytic core is different between the 8–17 (FIG. 6D) and the 17E deoxyribozymes (FIG. 6F), reflecting their different designs of the selection pool. Similar sequence motif was also observed in an RNA-cleaving deoxyribozyme (named Mg5) selected by Faulhammer and Famulok using 50 mM

histidine and 0.5 mM Mg^{2+} as cofactors (Faulhammer & Famulok, 1997). The homologous region in 31 out of the 44 sequenced clones had the sequence 5'-$TX_1X_2X_3AGCY_1Y_2Y_3ACGAA$-3' (SEQ ID NO:9), falling within the WCGAA-3' regime. The authors predicted a secondary structure different from the 8–17 or 17E motif based on chemical modification analysis. However, a structure containing a three-way junction similar to that of the 17E and 8–17 deoxyribozymes is consistent with the chemical mapping results.

Sequence Alignment and Structure Analysis of Co-DNA

The sequences of the *cis*-cleaving deoxyribozyme selected in the presence of Co^{2+} are more diverse than the Zn-DNA. They can be divided into three major classes based on sequence similarity (FIG. 3). There is no consensus sequence region among different classes. The secondary structure of each sequence class of Co-DNA was predicted with *DNA mfold* program. The minimal conserved sequence motif of class I Co-DNA includes a bulged duplex. The cleavage site is within the 13 nt single-stranded bulge. A 4 bp hairpin is also highly conserved and linked to the bulged duplex through 3 unpaired nucleotides. The folding of the sequences outside of this minimal motif was highly variable and resulted in structures with a wide range of stabilization energy.

The class II Co-DNA contains a sequence region (5'-ACCCAAGAAGGGGTG-3' (SEQ ID NO:10)) that was also found in an RNA-cleaving deoxyribozyme (termed G3) selected by Geyer and Sen (1997) (FIG. 7A and 7B). The minimal motif predicted for class II Co-DNA shows similarity to that proposed for the G3 deoxyribozyme as well. The G3 deoxyribozyme was believed to be fully active in the absence of any divalent metal ions. Copious use of divalent metal chelating agents, such as EDTA, and accurate trace-metal analysis of the reaction solutions were used to rule out the need of the G3 deoxyribozyme for contaminating levels of divalent metals. As mentioned earlier, the activity of class II Co-DNA was the same in buffer alone or with added Co^{2+} or Zn^{2+}, suggesting that this class of Co-DNA most likely contain the divalent metal-independent structure motif.

Effect of Metal Ions on the Activity of the *Cis*-cleaving Deoxyribozymes

ZnR17 and Co-18 were examined for their activity dependence on monovalent ions and divalent metal ions other than Zn^{2+} and Co^{2+}. In the presence of 1 mM EDTA and without added Zn^{2+} ions, no cleavage was observed with ZnR17 even after two days, strongly suggesting that divalent metal ions are required for the activity of ZnR17. Although the *cis*-cleaving Zn-DNA was selected in the presence of 500 mM NaCl, NaCl was actually inhibitory to enzymatic activity. With 0–2 M NaCl added to the reaction buffer (100 μM Zn^{2+}, 50 mM HEPES, pH 7.0), k_{obs} decreased with increasing NaCl concentration. The deleterious effect of NaCl was also manifested by lowered final percentage of cleavage products. For instance, only 50% of ZnR17 could be cleaved in the presence of 2 M NaCl even after long incubation times, while >95% of the DNA was cleavable in the absence of extra NaCl. Contrary to the Zn-DNA, the activity of Co-18 relies on NaCl and no cleavage was observed in the absence of NaCl. With 1 M NaCl, 8% of Co-18 molecules were cleaved within 5 min, while < 0.2% were cleaved in the absence of extra NaCl.

Although the deoxyribozymes were selected using either zinc or cobalt as cofactor, they are also active in other transition metal ions and in Pb^{2+}. The cleavage efficiency of ZnR17 followed the trend of $Pb^{2+} > Zn^{2+} > Mn^{2+} \sim Co^{2+} \sim Ca^{2+} > Cd^{2+} >> Ni^{2+} > Mg^{2+}$. It is noteworthy that the cleavage rate in Ca^{2+} was much higher than in Mg^{2+}, a similar trend was observed with the Mg5 deoxyribozyme. The order of Co-18 activity is as follow: $Zn^{2+} > Pb^{2+} \sim Co^{2+} > Ni^{2+} > Cd^{2+} \sim Mn^{2+} > Mg^{2+} \sim Ca^{2+}$. In general, both ZnR17 and Co-18 are more active in transition metal ions than in alkaline-earth metals, and higher activities were achieved with Pb^{2+}, Co^{2+} and Zn^{2+}. The preference of the selected deoxyribozymes for Co^{2+} and Zn^{2+} reflected their selection criteria. A similar trend ($Pb^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+}$) was also observed with all four RNA-cleaving deoxyribozymes selected in parallel by Breaker and Joyce using one of the four metal ions (Pb^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}) as cofactor (1995). The proposed secondary structures of the deoxyribozymes selected in Pb^{2+} and Mg^{2+} have been reported (Breaker & Joyce, 1994, 1995). No structure similarity was observed between ZnR17 and those RNA-cleaving deoxyribozymes.

Summary

Using *in vitro* selection technique, several groups of RNA-cleaving deoxyribozymes were isolated using Zn^{2+} or Co^{2+} as cofactor. No common sequence or structural features were observed between the Co(II)-dependent and the Zn(II)-dependent deoxyribozymes, in spite of the chemical similarities between these two transition metal ions. The deoxyribozymes selected in Zn^{2+} share a common motif with the 8–17 and the Mg5 deoxyribozymes isolated under different conditions, including the use of different cofactors. Both the Co-DNA and the Zn-DNA exhibited higher activity in the presence of transition metal ions than in alkaline earth metal ions, which are the most commonly adopted metal cofactors by naturally occurring ribozymes.

Example 2 – Deoxyribozyme as a Biosensor for Pb^{2+} Detection

This Example describes a fluorescence-based biosensor for the detection of Pb^{2+}. The biosensor utilizes a deoxyribozyme developed in Example 1 (termed 17E) combined with fluorescence technology to allow quantitative and real time measurements of catalytic activity. Because catalytic activity is dependent on Pb^{2+}, the biosensor provides real-time, quantitative, and sensitive measurements of Pb^{2+} concentrations.

Materials and Methods used in this Example

Oligonucleotides

The oligonucleotides were purchased from Integrated DNA Technology Inc. The cleavable substrate (Rh-17DS) is a DNA/RNA chimera with the sequence 5′-ACTCACTATrAGGAAGAGATG-3′ (SEQ ID NO:2), in which rA represents a ribonucleotide adenosine. This RNA base is replaced with a DNA base for the non-cleavable substrate (Rh-17DDS) (SEQ ID NO:11) used in the control experiment. Both substrates are covalently linked at the 5′ end with 6-carboxytetramethylrhodamin through NHS-ester conjugation. The deoxyribozyme (17E-Dy) is labeled at the 3′-end with Dabcyl via CPG phosphoramidite and has the sequence 5′-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3′ (SEQ ID NO:1). All the

oligonucleotides were purified by denaturing 20% polyacrylamide gel electrophoresis to ensure 100% labeling with the fluorescent dyes.

Fluorescence Spectroscopy

The enzyme-substrate complex was prepared with 50 nM each of 17E-Dy and Rh-17DS in 50 mM NaCl, 50 mM HEPES (pH 7.5) with a volume of 600 µl. The sample was heated at 90°C for 2 min and cooled to 5°C over 15 min to anneal the enzyme and substrate strands together. Steady-state and slow kinetic fluorescence spectra were collected with a SLM 8000S photon counting fluorometer. Polarization artifacts were avoided by using "magic angle" conditions. The steady-state emission spectra were collected from 570 to 700 nm (λ_{ex} = 560 nm). The time-dependent DNA enzyme catalyzed substrate cleavage was monitored at 580 nm at 2 s intervals. To initiate the reaction, 1 - 2 µl of concentrated divalent metal ion solution was injected into the cuvette using a 10 µl syringe while the DNA sample in the cuvette was constantly stirred.

DNA-based sensor of metal ions

An *in vitro* selected DNA enzyme from Example 1 (termed 17E) that is capable of cleaving a lone RNA linkage within a DNA substrate (termed 17DS) (FIG. 5) was chosen for use as a DNA-based, fluorescent biosensor of metal ions. Assays of this enzyme indicate a highly Pb^{2+} dependent activity with k_{obs} = 6.5 min^{-1} at pH 6.0 and $K_{apparent}$ = 13.5 µM [35]. The fluorosensor was constructed by labeling the 5'-end of the substrate with the fluorophore 6-carboxytetramethylrhodamin (TAMRA) and the 3'-end of the enzyme strand with 4-(4'-dimethylaminophenylazo)benzoic acid) (Dabcyl). Dabcyl serves as a universal fluorescence quencher. Steady-state fluorescence spectra were obtained by exciting the sample at 560 nm and scanning its emission from 570 to 700 nm.

When the substrate (Rh-17DS) was hybridized to the enzyme strand (17E-Dy), the fluorescence of TAMRA was quenched by the nearby Dabcyl (FIG. 8). Upon addition of Pb^{2+}, this quenching was eliminated and the fluorescence of TAMRA increased by ~400%. Little change in the fluorescence signal occurred with addition of Pb^{2+} to the substrate alone or to the complex of the enzyme and a non-cleavable all DNA substrate with identical sequence. These findings show that the change in fluorescent signal with Rh-17DS/17E-Dy results from a DNA enzyme-catalyzed substrate cleavage, followed by product release.

The substrate cleavage reaction was monitored in real time with fluorescence spectroscopy. Like the ratiometric, anisotropy, or lifetime-based method, kinetic fluorescence measurement is independent of sampling conditions such as illumination intensity and sample thickness (Oehme & Wolfbeis, 1997). In order to determine the selectivity of the catalytic DNA sensor, the fluorescence change (λ_{em} = 580 nm, λ_{ex} = 560 nm) of Rh-17DS/17E-Dy upon addition of nine different divalent metal ions that are known to be active toward DNA/RNA cleavage (FIG. 9A) was monitored. At the same concentration, Pb^{2+} caused the most rapid change in fluorescence with a rate of 380 counts · s^{-1} at 500 nM Pb^{2+}, pH 7.5. The sensitivity toward Pb^{2+} was > 80 times higher than other divalent metal ions (FIG. 9A). Remarkably, this trend of selectivity was maintained even under simulated physiological conditions containing 100 mM NaCl, 1 mM Mg^{2+}, and 1 mM Ca^{2+} (FIG. 9B). Furthermore, the signal response to Pb^{2+} was not affected by the presence of equal amounts of each of these divalent metal ions. Therefore, this DNA enzyme sensor is well suited for selective monitoring Pb^{2+} in the presence of other metal ions.

In addition to the selectivity of the DNA enzyme probe for Pb^{2+} over other metal ions, the range of Pb^{2+} concentrations which give rise to a fluorescent response is also important. As shown in FIG. 10A, the rate of fluorescence change increased with Pb^{2+} concentration up to 4 μM. The detection limit for Pb^{2+} is around 10 nM, ~ 50 fold less than the toxic level defined by the Center for Disease Control.

Example 3 – DNA chip comprising an array of nucleic acid enzymes

This prophetic example describes the production of and use of a DNA chip for sensing ions, in particular heavy metal ions.

The first step towards the application of deoxyribozymes in heavy metal sensing is to obtain various deoxyribozymes with different metal specificity and affinity. *In vitro* selection will be carried out to isolate a variety of deoxyribozymes. A detailed description of the selection protocol can be found in Example 1. Each family of deoxyribozyme will be specific for different divalent metal ions (e.g. Pb^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, etc). Within each family, different sequences will have different affinities of the specified metal ion.

These deoxyribozymes and their substrates will then be arrayed onto a DNA chip with one dimension for metal ion specificity and the other for affinity of the corresponding metal (FIG. 11). The enzyme strands immobilized on the chip at 3'-ends can be synthesized on the chip using photolithographic methods (Fodor *et al.*, 1991; Pease *et al.*, 1994) or can be synthesized off-chip and then attached to the chip using various methods (Joos *et al.*, 1997; O'Donnell-Maloney *et al.*, 1997; Guschin et al., 1997). The 5'-end of the enzyme strand will be labeled with a fluorophore. The 3'-end of the substrate strand will be labeled with a fluorescence quencher, which can be a fluorescent or non-fluorescent moiety. For example, guanidine base is an efficient quencher of fluorescein.

Hybridization of the enzyme and substrate will result in the quenching of the donor fluorescence. Upon exposure to the sample containing the active metal ion, the substrate will be cleaved and products will dissociate, resulting in strong fluorescence of the dye attached to the enzyme strand. The metal ion species can be qualitatively identified based on the metal specificity of different families of deoxyribozymes. A hypothetical sample result is shown in FIG. 11B. The pattern of fluorescence intensity shows that there are three kinds of metal (M1, M4, and M6) in the sample.

The concentration of the metal ion under inspection can be quantified with deoxyribozymes with different metal affinity. Given a certain concentration of the metal ion, different sequences within the same family will have different cleavage efficiencies due to their different threshold in response to the metal concentration. The metal concentration applied may exceed the saturation concentration of those having higher affinity; therefore full cleavage will occur within a certain time and present strong fluorescence. On the other hand, the substrates of those with lower affinity will only be partly cleaved and emit weaker fluorescence. The sample hypothetical result shown in FIG. 11B shows high (c1), medium (c4), and low (c6) concentrations of M1, M4, and M6, respectively.

The fluorescence patterns with respect to different deoxyribozyme sequences will be compared with standard calibration maps. After de-convolution of the

fluorescence pattern, direct information can be obtained about the identity and concentration of metal ions in the samples.

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